e.g., acidosis.

This great difference in chemical structure of compounds capable of producing the same deformity is of interest in comparison with the findings of Wilson on the teratogenicity of azo dyes chemically related to trypan blue(6). Here, it was found that a total dose of 135 mg/kg of trypan blue produced 49% deformed fetuses, while the same amount of a closely related structural isomer, Evans blue, resulted in a deformity rate of only 14%.

It should be pointed out that the amount of dichlorphenamide (in mg/kg) used to produce this teratogenic effect is more than 100 times greater than that used in the treatment of human patients.

Summary. Administration of very high doses of dichlorphenamide, a carbonic anhydrase inhibitor, in the diet of rats during pregnancy is associated with the production, in some of the offspring, of a deformity consisting of a postaxial defect of the right forelimb. This is not associated with any other demonstrable anatomical abnormalities. This deformity appears to be the same as that caused by acetazolamide, a compound of quite different chemical structure which is also a potent carbonic anhydrase inhibitor.

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Effect of Hypervitaminosis D Upon the Phospholipids of Metaphyseal And Diaphyseal Bone.* (32353)

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Previous work has shown that the hypervitaminotic D state affects the quantity of lipids which can be extracted from the matrix of rat bone(1,2). Hypervitaminosis D increased the total lipids, phospholipids, triglycerides, cholesterol, cholesterol esters, and total fatty acids of the diaphyseal and metaphyseal long bones. The greatest change was in the phospholipid fraction. There is no information available in the literature on the nature of the phospholipids of long bones or on the mechanism of action of vitamin D in lipid metabolism. Consequently, the following experiments were carried out to determine which phospholipids were present, whether the effect of vitamin D on phospholipid metabolism is confined to a single phospholipid or concerns all major phospholipid components, and to ascertain whether the changes are the result of increased synthesis or decreased break-down.

Materials and methods. Male hooded rats of the R.V.H. strain averaging 110 g were maintained on a complete synthetic laboratory diet (Purina Labina). The hypervitaminotic D group received 15,000 international units of vitamin D_2‡ dissolved in sesame oil per 100 g of body weight daily. The control animals were dosed with an equal volume of sesame oil. Between days 14 and 18, 11 control

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‡ Vitamin D_2 (calciferol) kindly supplied by N. V. Phillips-Roxane Co. The vitamin D was dissolved in a small amount of absolute alcohol and diluted with sesame oil so that the concentration of vitamin D in sesame oil was 15,000 units/ml.

and 12 vitamin D treated animals were decapitated and the humeri, femora and tibiae removed and cleaned of soft tissue. The epiphyses were pried off and the marrow of the long bones was removed by means of a syringe. The bones were then prepared and incubated in Krebs-Ringer-Bicarbonate (pH 7.4) according to the method of Deiss, Holmes, and Johnston(3). Ten microcuries of P^{32} as $NaHPO_4$ were added to each flask. After 6 hours of incubation, the bone fragments were removed, washed immediately with cold saline, and lyophilized. They were ground in a Spex Grinder in the cold for 2 minutes and the lipids were extracted and washed according to the method of Folch *et al*(4). This procedure was found to remove all but .05% of added inorganic P^{32}. The bones of the right extremities were kept separate from those of the left and prior to incubation the latter bones were placed in a steam bath for 5 minutes. The uptake of P^{32} into the phospholipids of the steamed bones never exceeded 1% of that of the non-steamed ones. Ash weight was determined by ashing an aliquot of dried fat-free bone in a muffle furnace at 680°C for 48 hours. The fraction of organic matter was calculated by subtracting the per cent ash from the total dry, fat-free weight. Lipid phosphorus was determined on an aliquot of the chloroform-methanol extract by the method of Marinetti(5) and phospholipid calculated by multiplying the phosphorus content of the lipid extract by a factor of 25(6). An aliquot of the chloroform-methanol extract was counted in a Packard Tri-Carb liquid scintillation counter and suitable corrections were made for background, decay, and quenching.

An additional group of 6 control and 6 hypervitaminotic D animals was maintained for 24 days, at which time they were sacrificed and their bones were removed, prepared and the lipids extracted as described above. Aliquots of the extracts were applied to thin-layer chromatograms using activated silica gel-G and the solvent system of Wagner *et al*(7), was used for chromatographic separation. Identification and quantification of the individual phospholipid classes were carried out by the method of Blecher and Abramson(8). Total phospholipid, ash, and per cent of organic content were determined as outlined above. The use of 2-dimensional thin-layer chromatography according to the method of Skidmore and Entenman(9) failed to reveal any significant additional phospholipids.

Results. As can be seen in Tables I and II, the rats which had been receiving high doses of vitamin D failed to gain weight at the same rate as the controls and there was a statistically significant increase in the organic fraction of their bones, as has been shown before(10). Table I shows that there is an increase in the amount of phospholipid of these bones whether this value is expressed as mg/g of bone or mg/g of the organic fraction. The P^{32} uptake was significantly increased whether it was expressed as counts per g bone or counts per mg of phospholipid. Table II shows that lysolecithin, sphingomyelin, lecithin, and phosphatidylethanolamine constitute the major phospholipids of the matrix of diaphyseal and metaphyseal bone. One unidentified phospholipid making

TABLE I. P^{32} Uptake by Bones of Hypervitaminotic D Animals 14-18 Days on Treatment.

	Control (11 animals)	Hypervitaminosis D (12 animals)
Final wt (g)	170 ± 4.2	116 ± 6.3 ***
Bone—Ash (% dry, fat-free wt)	65.3 ± 1.47	58.5 ± 1.34***
Bone—Organic fraction (% dry, fat-free wt)	34.7 ± 1.45	41.5 ± 1.34***
Phospholipid—Bone (mg/g)	1.97± .22	3.24± .37***
Organic fraction (mg/g)	5.64± .35	7.93± .58**
P^{32} uptake—Counts/min/g bone	7629 ±1502	15,701 ±2041 **
Counts/min/mg phospholipid	3441 ± 764	6,418 ±1460 **

*** Significance at .1% level ($p < .001$).
** " " 1% " ($p < .01$).
* " " 5% " ($p < .05$).

± Standard error

TABLE II. Phospholipids of Bones by Hypervitaminotic D Animals 24 Days on Treatment.

	Control (6 animals)		Hypervitaminosis D (6 animals)	
Final wt	182 ± 5.2		121 ± 5.8 ***	
Bone—Ash (% dry, fat-free wt)	65.0 ± 1.37		58.4 ± 1.46***	
Bone—Organic fraction (% dry, fat-free wt)	35.0 ± 1.35		41.6 ± 1.47***	
	mg/g organic fraction	% of total phospholipid	mg/g organic fraction	% of total phospholipid
Total phospholipid	7.03 ± .42		11.60 ± .64***	
Lecithin	3.63 ± .24	51.6	5.99 ± .28***	51.1
Phosphatidylethanolamine	2.22 ± .18	31.5	3.01 ± .20**	26.5
Sphingomyelin	.53 ± .09	7.6	1.19 ± .11**	10.3
Lysolecithin	.30 ± .07	4.3	.60 ± .09	5.1
"X"	.16 ± .04	2.2	.46 ± .06	4.1

*** Significance at .1% level ($p < .001$). ± Standard error
** " " 1% " ($p < .01$).
* " " 5% " ($p < .05$).

up 2-4% of the total also was found. It travelled between phosphatidylethanolamine and the solvent front and contained neither amino acids nor sugars. It can be seen that the increase in phospholipids resulting from excess vitamin D is the result of a rise in all phospholipids, rather than a selective increase in any one and that it is in all probability due to an increased synthesis based on the P^{32} incorporation.

Discussion. The recovery of lipid phosphorus applied to thin-layer chromatograms was between 85 and 90% and was quite constant. All extracts were compared with known standards whose recoveries also were within this range. Two faint additional spots were found on the 2-dimensional thin-layer chromatograms and these corresponded to very small amounts of phosphatidic acid and cardiolipin. No other unidentified fractions were found and it is therefore felt that the major phospholipids of the matrix of metaphyseal and diaphyseal rat bone as extracted by the Folch procedure(4) have been described.

An interrelation between calcification and lipids has been observed by Irving(11). He found a sudanophilic material at the zone of calcification of the epiphyseal line of calves and suggested that lipids and particularly phospholipids may be involved in bone formation. Johnson(12) also has implicated lipids in the series of events which lead to calcification of bone matrix in many tissues. Using histochemical techniques, he has shown that neutral lipids are present between the osteoblast and the calcifying front. After the affinity for neutral lipid stain disappears, the tissues will stain for phospholipid and this affinity disappears as calcification occurs. These histologic observations are strongly suggestive that phospholipids may play a role in osteogenesis. It is well established that an excess of vitamin D has profound effects on the chemical composition and morphology of skeletal tissue(10,13,14). Recently it has been shown that the lipids of bone are increased in hypervitaminosis D and that the most marked increase occurs in the phospholipid fraction(1,2). It is possible that the involvement of vitamin D in skeletal metabolism is through its role in phospholipid metabolism. It is known that in hypervitaminosis D there is a significant increase in the organic fraction of bone and that also the collagen and mucopolysaccharide content of this fraction is increased(10). Therefore it is not too surprising that the lipid content of bone also is elevated since it too is associated with the organic fraction. Further, there is accelerated bone production in the medullary cavities of long bones with large quantities of uncalcified osteoid occurring about the original trabeculae(13,14). It is unlikely that calcification cannot keep up with the increased rate of osteogenesis because there is,

indeed, a hypercalcemia in this condition. The present findings of an increased rate of phospholipid formation as measured by the uptake of P^{32} are consistent with the increased rate of bone production. Thus, in hypervitaminosis D, since the rate of matrix formation is greater than normal, it may be that the rate of calcification is inhibited by the presence of excess lipids and in particular phospholipids. It may be that calcification cannot occur until the phospholipids are removed. This could account for the decreased mineral content of the skeleton in hypervitaminosis D. It seems unlikely that any particular phospholipid is specifically involved since the present study showed that all phospholipids were increased proportionately.

Thompson and DeLuca(15) and Hoyosha (16) have shown that the vitamin D-deficient state leads to a diminished incorporation of P^{32} into the phospholipids of rat mitochondria from intestine and kidney but not from liver. The present work clearly indicates an increased incorporation of P^{32} into the phospholipids of bone in the presence of excess vitamin D. Thus, vitamin D status of the animal has significant effects on the phospholipid metabolism of tissues which are influenced by this vitamin, *i.e.*, kidney, intestine and skeleton.

Summary. The phospholipids of the diaphyseal and metaphyseal portion of the long bones of rats have been analyzed. Lecithin, phosphatidylethanolamine, sphingomyelin and lysolecithin constitute the major phospholipids with small amounts of cardiolipin and phosphatidic acid also being present. Hypervitaminosis D was found to cause a significant increase in all phospholipids without a selective action on any one component. The incorporation of P^{32} into the phospholipids was found to be increased indicating an increased synthesis rather than a decreased break-down. It is suggested that the accumulation of lipid material may be related to the failure of the osteoid in hypervitaminosis D to calcify properly.

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Nutritional Studies with the Guinea Pig. VIII. Thiamine. (32354)

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In a previous report from this laboratory, the effects of thiamine deficiency in the guinea pig were briefly described(1). The present report gives results of additional experiments designed to determine the approximate requirement of this animal for thiamine.